REDOX INDICATOR PATTERNS IN RELATION TO ECHINODERM EXOGASTRULATION.

I. OXIDATION PATTERNS

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In a recent paper on gastrulation in the echinoid, *Dendraster excentricus,* following trypsin treatment, A. R. Moore (1952, p. 45) found that gastrulation occurred, even though the hyaline membrane had been removed by trypsin. Concerning this result he said, "This proves that the tensile force of the outer membrane is not an agent in gastrulation. The evidence leads to the conclusion that the process of infolding is the result of properties inherent in the vegetal plate" (Moore, 1952, p. 45). This conclusion, together with certain data concerning redox indicator patterns in echinoderm development, suggested to the present writer that further investigation of indicator patterns, particularly in relation to exogastrulation, might give some evidence concerning conditions which determine or are associated with entogastrulation and exogastrulation. The earliest observations on echinoderm indicator pattern concerned only patterns of intracellular reduction by decrease of oxygen content in the external medium after staining by oxidized "vital" dyes. As the title indicates, the present paper concerns only oxidative patterns: the data concerning reduction patterns are not complete, particularly as regards exogastrulation. It is hoped that, not only the earlier, but also more recent data concerning reduction patterns may be considered in a later paper. In the following data concerning oxidative patterns, particular attention is given to a feature of these patterns, not fully recognized in earlier work in consequence of the preoccupation with reduction patterns.

Material and Methods

The echinoids, *Dendraster excentricus* and *Strongylocentrotus purpuratus*, and the asteroid starfish, *Patiria miniata,* have served as material, either at the Hopkins Marine Station, Pacific Grove, or at Palo Alto after transportation of the animals under slight refrigeration, and development in most cases at temperatures ranging from 18 to 20° C., or in some lots at approximately 13° C., or even 8–10° C. with *Dendraster,* during earlier stages. In *Dendraster* and *S. purpuratus* good fertilizations were obtained for three days after transportation to Palo Alto with *S. purpuratus* and for five days with *Dendraster* from animals kept in a refrigerator at 10–15° C., covered by seaweed or wet paper toweling and with a small amount of water in the container. Starfish material for the present purpose was fertilized

at Pacific Grove and brought to Palo Alto at once. *S. purpuratus* is more sensitive to environmental conditions than the other forms used.[1]

For intracellular oxidation a modification of the indophenol or Nadi reaction with very low concentrations of the two reagents, para-amino-dimethyl aniline (dimethyl-paraphenylene diamine) and α-naphthol as described repeatedly in detail in earlier papers (*e.g.,* Child, 1944, 1953b). Intracellular oxidation of the two reagents is catalyzed by an oxidase, regarded by some as cytochrome oxidase. With the low concentrations of the reagents and without the necessity of using alkali to dissolve the naphthol, the material is not killed at once and motile stages remain active until intracellular concentrations of indophenol become high. This modification of the Nadi reaction makes directly visible very slight regional differentials even more clearly in most cases than the catalyzed intracellular oxidations of dye solutions reduced by non-toxic concentrations of sodium hydrosulphite. This is largely due to the brilliant blue color of intracellular indophenol, even in very low concentrations.

In addition to the indophenol reaction various dyes, chiefly diazine green, methylene blue and in some cases Nile blue sulphate, reduced in external solution by sodium hydrosulphite ($NaHSO_2$ or $Na_2H_2S_2O_4$) were used. Minute amounts of hydrosulphite, fractions of a milligram, are sufficient to reduce one ml. of methylene blue 1/10,000 and the lower concentrations used of diazine green and Nile blue sulphate. This reducing agent has great advantages over the highly toxic reducing agents used in earlier work with redox indicators: those sometimes led to errors in results by retarding or completely inhibiting reduction in the most sensitive regions. Hydrosulphite is not appreciably toxic in concentrations and with exposure periods far above those required for dye reduction. Intracellular oxidation will occur in completely reduced external hydrosulphite dye solution if there is no great excess of hydrosulphite. The indophenol reaction and intracellular dye oxidation have been used repeatedly and results observed in many hundreds of individuals during the breeding seasons of most years from 1947 on.

Figures are essentially optical sections along the polar axis. Different magnifications are used in the three species. The egg and developmental stages of *S. purpuratus* are considerably smaller than those of the other forms and those of the starfish are larger than the *Dendraster* stages. Sizes of figures do not indicate the actual differences in size in the three forms but have been chosen chiefly in order to show the gradient patterns clearly. Figures of *S. purpuratus* are considerably more, those of the starfish less, magnified than the actual differences in size would require for representation. Intracellular indophenol reaction and dye oxidation are indicated by shading and by arrows pointing from regions of more rapid to those of less rapid reaction. Arrows external to the inhibited ectodermal regions of exogastrulae indicate only differentials along the polar axis without reference to differentials in the cell-wall. In the more extreme types of exogastrulae the ectoderm is more inhibited than entoderm and these differentials are slight and are indicated by short arrows, or they may be entirely absent. In all except one figure the entodermal differentials are indicated by shading.

[1] The kindness of the Director and staff of the Hopkins Marine Station in providing material and laboratory facilities, and in many cases for transporting the animals to Palo Alto, is gratefully acknowledged.

Earlier Data Concerning Oxidation Patterns

The sand dollar, *Dendraster excentricus,* was the material for the earliest observations on oxidation patterns, and the indophenol reaction was used. Only the more important features of these patterns were discussed and these only briefly (Child, 1941b). In later cleavage stages and in blastulae of normal[2] development, the reaction was distinctly differential, decreasing in rate basipetally from the apical[3] region. In the gastrula and probably earlier, a ventrodorsal reaction gradient also became visible with ventral ectodermal region oxidizing more rapidly than dorsal. Within the blastocoel, mesenchyme apparently reacted more rapidly than entoderm, but with progress of invagination an entodermal gradient, decreasing basipetally from the tip of the archenteron, developed and rate of reaction at the tip increased still further with coelom formation. In still later stages the tips of the developing oral lobe and anal arms became the "high" ends of new ectodermal gradients. As the ciliated band developed, its cells also showed increase in reactivity above that of the general ectoderm. With gradual progress of starvation in the fully developed plutei the gradient differentials decreased, and before death almost completely disappeared. In these earlier data on oxidation gradient patterns it was not determined whether a differential in rate of oxidation was present between blastocoelar and outer surface of the cell-wall of the blastula and early gastrula, although it had been determined still earlier that dye reduction decreased in rate from the blastocoelar to the external surface (Child, 1936a, 1936b) and it had been suggested that this was probably due to lower oxygen content in the blastocoel than outside.

In a later, more extended study of indophenol reaction and reduction in normal *Patiria* development (Child, 1944) the rate of the polar indophenol oxidation gradient was found to decrease basipetally from the apical region in later oöcytes, cleavage stages, blastulae and early gastrulae. With progress of invagination a new oxidation gradient developed in the entoderm, as in *Dendraster,* with decrease in rate basipetally. A ventrodorsal oxidative gradient seemed to be visible in some late blastula stages, but is not indicated in the figures of these stages since it was difficult to determine when it first appeared. With further development it became more clearly visible and with coelom and stomodeum development other local oxidative patterns appeared. That paper, like the earlier studies of indicator patterns, in echinoderm development, was concerned primarily with polar pattern, and the indophenol reaction was allowed to continue until the polar and later local differentials became distinct, without much attention to the earlier stages of the reaction. However, it was indicated by the course of the arrows in certain figures, *e.g.,* Figures 11, 13–17, 19 and 20 (Child, 1944), that reduction in the cell-wall of blastulae and early gastrulae progressed from the blastocoelar surface outward. This differential was still regarded at that time as probably resulting from lower

[2] It is perhaps unnecessary to note that "normal" development indicates merely the range of variations occurring under those conditions which we regard as natural. In its origin and determining factors it does not differ in any way from the experimental modifications of development under other conditions.

[3] The terms "apical" and "basal" are used for earlier developmental stages as less awkward than "animal" and "vegetal" or "vegetative" and as permitting use of the terms "basipetal" and "acropetal" in description of gradient patterns.

oxygen content in the blastocoel than outside and therefore of minor importance in development. It was not even determined whether a differential in indophenol reaction, an oxidative differential, appeared in the cell-wall in early stages of the reaction. As will appear in the present paper, an oxidase differential is present in the cell-wall of the blastula and early gastrula. The failure to observe it in this earlier investigation of indicator pattern is an interesting example of the influence which a preconceived opinion may have on observation.

Redox indicator patterns of echinoderm exogastrulae have not been thoroughly studied. In the first attempt to learn something about indicator patterns of exogastrulae, only differential reduction of vital dyes was determined following staining by oxidized dyes with oxygen decrease in the external medium. These data are to be considered in a later paper. Patterns of intracellular oxidation of indicators have been determined only in *Dendraster* and only by means of the indophenol reaction and have been recorded only briefly (Child, 1941b). In that paper it was stated that in exogastrulae with enlarged and elongated entoderms indophenol reaction progressed from the entodermal tip toward the ectoderm. It was also noted that in the thick-walled blastulae which occur in exogastrulating agents and usually become exogastrulae, if not too much inhibited for further development, the cell-wall gradient progressed from the blastocoel outward, but the cell-wall oxidative gradient after actual evagination and elongation of the entoderm was not considered. In the less extreme types of exogastrulae in which the ectoderm approaches or attains pluteus form, the ectodermal oxidative pattern is essentially like that of the normal pluteus. In the more extreme forms of exogastrulae the ventrodorsal oxidative gradient is entirely absent. The polar gradient is present, at least in the evaginated entoderm, but may be completely absent in the ectoderm or that part of it which has not been entodermized. There again, it was not determined whether a cell-wall oxidative gradient was present in these elongated exogastrular entoderms.

In *Patiria* the indophenol reaction gradient, the oxidative gradient pattern, was determined only for normal development, and information concerning oxidative patterns of exogastrulae has been completely lacking up to the present.

Oxidation Patterns of Normal Blastulae, Early Gastrulae and Exogastrulae of Dendraster

The polar pattern of the indophenol reaction and of oxidation of vital dyes reduced by sodium hydrosulphite in the normal blastula is a distinct gradient decreasing basipetally from the apical region (Fig. 1, *A*), like those of other echinoderms, so far as known (Child, 1941b, 1944, 1953b). However, the most interesting feature of the blastula pattern and one which has been largely neglected, as already noted, is the cell-wall gradient, decreasing from the blastocoelar to the external surface. It becomes visible with oxidation of vital dyes as well as with the indophenol reaction. Since this is an oxidative gradient pattern it evidently cannot be due to lower oxygen content in the blastocoel than outside. The immigrating mesenchyme cells react essentially like adjoining cells of the cell wall. The same polar and cell-wall pattern are present in the basal region and in the invaginating entoderm of the early gastrula (Fig. 1, *B*), but the ectoderm soon becomes so thin that presence of a cell-wall gradient in it becomes uncertain, *i.e.*,

the differential in the thin layer, if present, is not sufficient to be clearly visible, except in an apical thickening, as in Figure 1, *B*. In many individuals the cell-wall gradient still seems to be barely visible but it is not consistently distinguishable in all as development progresses. In general, the cell-wall gradient is most clearly visible in early stages of the indophenol reaction and dye oxidation. As intracellular concentration of indophenol or dye increases, it becomes progressively less distinct, and finally almost or quite indistinguishable.

Figure 1, *C* is a *Dendraster* exogastrula from a lot subjected to rather extreme change from low to high temperature. This and several other lots were kept after

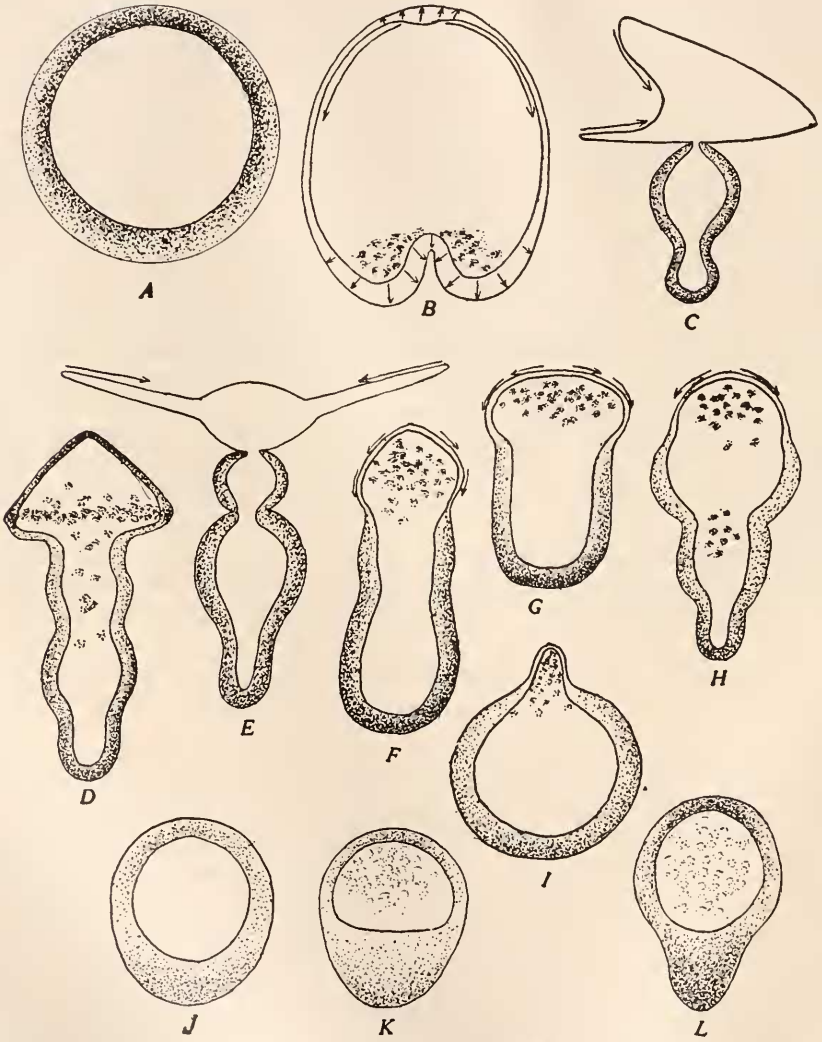

FIGURE 1. Oxidation patterns of *Dendraster excentricus: A*, normal blastula; *B*, normal early gastrula; *C-I*, LiCl exogastrulae; J-L, sodium azide pre-exogastrulae and early exogastrulae. For data concerning experimental conditions see the text.

fertilization at 8–10° C. and after 28 hours had developed to medium blastula stages. At this stage they were brought to a temperature of 20–22° C. for further development without any inhibiting agent. In the various lots this procedure resulted in exogastrulae ranging from an estimated 30 to 80–90 per cent, mostly of the less extreme types like Figure 1, *C*, with some degree of ventrodorsality and often approach to the pluteus form in the ectoderm. In the entoderm of Figure 1, *C* the polar gradient decreases from the tip toward the ectoderm and the cell-wall gradient from the external surface to the blastocoel, *i.e.*, opposite in direction to the cell-wall gradient in normal development.

Figures 1, *D–I* are various degrees of exogastrulation with exposure from the 4–8 cell stage to LiCl *M*/40 or *M*/50 for 24 hours or in Figure 1, *I*, 48 hours in *M*/50, followed by a day or two in water, at a temperature of 18–20° C. In *D*, as in *C*, the thin ectoderm is merely indicated in outline and gradient patterns by arrows. In the exogastrulae of *E-I* of Figure 1, the polar entodermal gradient decreases from the entodermal tip toward the ectoderm, and the cell-wall gradient from the external surface inward. This cell-wall pattern has been observed in many hundreds of *Dendraster* exogastrulae, the gradient of normal development, from the blastocoel outward, in none. In Figure 1, *E* the ectoderm is somewhat less inhibited than in *F-I* and its polar gradient is indicated by shading. In *I*, the ectoderm is almost absent and without distinguishable gradient.

Figures 1, *J*, *K*, and *L*, were in water for one or two days after 23 hours in sodium azide *M*/350 from the 8-cell stage at 20–22° C. throughout. In these, little or no differential recovery occurred after return to water. They are forms with thickened entodermal region, slight indication of evagination of entoderm and are pre-exogastrulae, or in *L* a slight degree of exogastrulation. In these, and large numbers of others in the same or lower azide concentrations, the polar entodermal gradient decreased from the tip toward the ectoderm, the cell-wall gradient from the external surface inward, as in LiCl exogastrulae, though usually with less differential than with LiCl. In these three examples of azide differential inhibition, the ectodermal cell-wall has remained relatively thick and the usual polar gradient was present in it, decreasing from the apical region, but with rather slight differential. A cell-wall gradient was also distinguishable in these ectoderms, decreasing from the blastocoel outward, *i.e.*, the same as in normal development. If reversal in direction of the entodermal cell-wall gradient is associated in any way with exogastrulation, reversal of the ectodermal cell-wall gradient is not to be expected when the course of its development is not altered. After a day or two in water entodermal dissociation, usually external, began in many individuals like Figures 1, *J–L* and the indophenol reaction was usually more rapid in the dissociated and apparently cytolyzed or cytolyzing cells than in intact entodermal cells.

The *Dendraster* blastula and early gastrula stages of Figures 1, *A* and *B* represent patterns observed in hundreds of control individuals. Their presence has been repeatedly confirmed by Dr. Olin Rulon. Also the entodermal patterns of Figures 1, *C–L* are selected cases only insofar as they are intended to show different degrees of exogastrulation and approaches to it. In all cases of the temperature, LiCl and azide forms, the entodermal cell-wall gradient is reversed in all degrees of evagination of entoderm and the approaches to it.

Oxidative Patterns of Blastulae and Exogastrulae of Strongylocentrotus purpuratus

Developmental stages of *S. purpuratus* are smaller than those of *Dendraster* and *Patiria* and the cell-walls become so thin in the course of development that this form is somewhat less favorable material than the others, particularly as regards the cell-wall gradient. Moreover, it has seemed from earlier use of indicators that gradient pattern in this form has somewhat less differential than in the other two echinoderms. The polar gradient and the ventrodorsal gradient, when not obliterated by inhibiting conditions, are distinct in developmental stages, but after the cell-walls have become thin it is sometimes difficult to determine with certainty whether a cell-wall gradient is present. The inhibiting agent used for exogastrulation also decreases gradient differentials, but after return to water and more or less differential recovery, they may again increase to some extent. As starvation of the larvae progresses, the differentials of oxidative gradient pattern decrease and before death usually almost disappear. However, in normal development and in exogastrulae before the cell-walls have become very thin the differentials of gradient pattern are similar to those of *Dendraster*, though apparently less "steep."

In Figure 2, *A* the polar and cell-wall gradients of indophenol reaction in the normal blastula are indicated. The oxidation pattern of dyes reduced by hydrosulphite is similar. Earlier stages of *S. purpuratus* were also examined in an attempt to determine at what stage the cell-wall gradient became distinguishable. Often repeated examination of the earlier cleavage stages indicated that this gradient, decreasing from the blastocoelar surface, became distinguishable soon after a distinct blastocoel appeared, *i.e.*, about at 32- or 64-cell stages or perhaps somewhat earlier. At these stages the differential is slight and becomes distinguishable only in the earlier stages of intracellular oxidation. It has often seemed to be more distinct in the basal regions of the embryos, perhaps only because the cells of that region are larger than others. Certainly in later stages there is less differential basally than in the apical region (Fig. 2, *A*).

Also, often-repeated attempts have been made to determine whether the same cell-wall gradient is still present during invagination of the entoderm in normal gastrulation, as it is, at least in early stages, in *Dendraster* (Fig. 1, *B*). As invagination progresses, however, the cell-wall becomes so thin that, although in many cases the cell-wall gradient, decreasing from the blastocoel outward, seemed to be present in early stages of intracellular oxidation, its presence was still regarded as questionable.

In Figure 2, *B*, after 20 hours in LiCl *M*/20 at 18–20° C. without return to water, there is a slight basipetal polar gradient and in the apparently beginning evagination of the basal region, the prospective entoderm, indophenol and dye oxidations decrease from the entodermal tip, *i.e.*, from the outer surface inward, a reversal of th normal pattern. Figure 2, *C* is a somewhat similar case, also after 20 hours in LiCl *M*/20 at 18–20° C. Here a double gradient is present in the entodermal region, on the blastocoelar side a slight gradient decreasing from the blastocoel for a short distance, and externally another slight gradient, decreasing from the tip of the entodermal region and from the external surface inward. In this individual complete reversal of the entodermal cell-wall gradient has not yet occurred. The mesenchyme cells oxidize about as rapidly as, perhaps slightly more rapidly

than, the adjoining entoderm. The ectodermal polar gradient decreases basipetally and the cell-wall gradient remains as in normal development.

Figure 2, *D* represents an exogastrula two days in water after one day in LiCl *M*/40. In the thicker distal entoderm intracellular oxidation decreases from the entodermal tip and the cell-wall gradient from the outer surface inward. In the proximal entodermal segment a polar gradient is present with decrease toward the ectoderm, but the cell-wall has become so thin that presence of a cell-wall gradient is uncertain. In the ectoderm the polar and cell-wall gradients remain as in normal development.

The exogastrulae of Figures 2, *E* and 2, *F* were two days in water after 25 hours in LiCl *M*/50. Th entodermal polar gradient decreased from the tip, the cell-wall

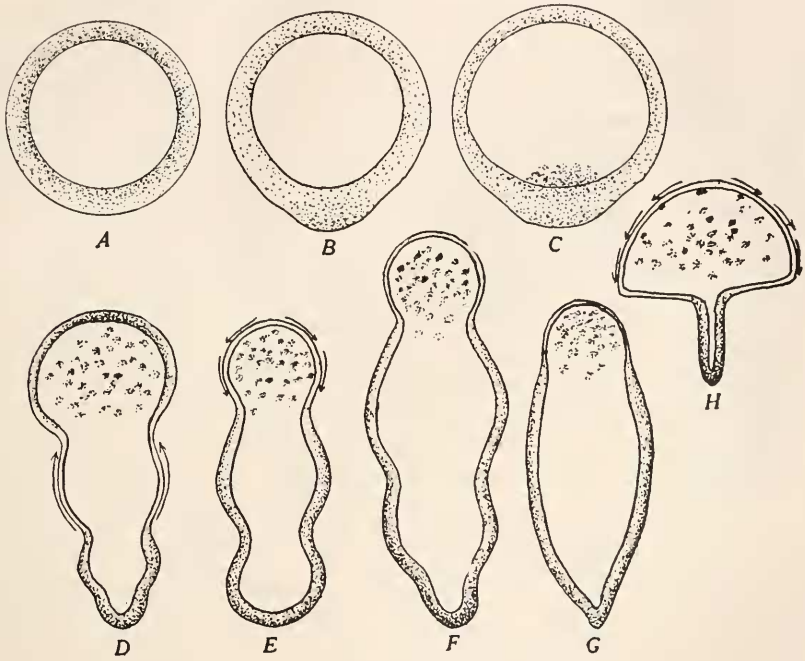

FIGURE 2. Oxidation patterns of *Strongylocentrotus purpuratus: A,* normal blastula; *B, C,* pre-exogastrulae; *D–H,* LiCl exogastrulae, some of mesenchyme cells becoming pigmented; data concerning experimental conditions in text.

gradient from the external surface inward. In the case of Figure 2, *E* the ectoderm retained a slight polar gradient but no cell-wall gradient was distinguishable; in the exogastrula of Figure 2, *F* no ectodermal gradient was visible.

The exogastrula of Figure 2, *G*, with the same treatment as *E* and *F*, was more inhibited than those, but with the same entodermal pattern and without distinguishable gradient pattern in the ectoderm. Figure 2, *H* from the same lot of eggs, with the same treatment and in the same container as *E–G*, is an example of the regional differences in inhibition which may occur in the same lot. The entoderm is more, the ectoderm less inhibited than in most animals of the lot, but the entodermal oxi-

dation pattern is the same as in *E–G*. The ectoderm is thin, and only a polar gradient is distinguishable.

Similar oxidative patterns have appeared in material two days in water following two days in sodium azide *M*/800 and *M*/1000 at the same temperature range as the preceding cases. Since these forms presented nothing new, additional figures are regarded as unnecessary. Thus far no exogastrulae of *S. purpuratus* without reversal of the cell-wall oxidative gradient have been seen, though in later stages of exogastrular life the entodermal cell-wall often becomes so thin that presence of a cell-wall gradient becomes questionable. Also in pre-exogastrulae and cases of slight evagination double entodermal cell-wall oxidative gradients are not infrequently present, as in Figure 2, *C*.

Oxidative Patterns of Blastulae and Exogastrulae of Patiria

Of the three echinoderms included in this paper, *Patiria* is perhaps the most interesting as regards the cell-wall gradient and its alterations in relation to exogastrulation. The following data constitute the first evidence that an oxidation gradient, as well as a reduction gradient, is present in the cell-wall of *Patiria*. In the normal blastula and early gastrula the gradient pattern is like that of the echinoids. The polar oxidation gradient decreases basipetally from the apical region and at all levels the rate of indophenol reaction and dye oxidation decreases from the blastocoel outward in the cell-wall (Fig. 3, *A*). As the entoderm invaginates, a new gradient, decreasing from the entodermal tip, appears, as in the echinoids (Fig. 2, *B*; see also Child, 1944, Figs. 21–23). The entodermal cell-wall gradient persists, with decrease from the blastocoel outward; as the ectoderm becomes thin, the polar gradient is still visible, but in the thin cell-wall of the ectoderm of later stages it becomes impossible to determine whether a cell-wall gradient is still present.

The forms of Figures 3, *B–I* were in LiCl *M*/50 for 20 hours from late cleavage to early blastula stages. It was noted earlier (Child, 1953a) that these stages have been found more favorable for exogastrulation than exposure to the inhibiting agent in early cleavage. All developed at 18–20° C. The forms of Figures 3, *B* and *C* were a day in water after 20 hours in LiCl. In Figure 3, *B* the thickened, slightly evaginated entoderm shows an oxidation gradient decreasing from the outer basal surface and also a slight gradient decreasing from the blastocoelar surface of the entoderm. The ectodermal cell-wall gradient decreases from the blastocoel outward and the slight gradient on the blastocoelar side of the entoderm is merely persistence of a very slight differential of this gradient. In Figure 3, *C* the entodermal evagination has progressed much further and the cell-wall gradient decreases from the entodermal tip and from the external surface inward. In the thin ectoderm only a slight polar gradient is present as indicated. The exogastrulae of Figures 3, *D* and 3, *E* were two days in water after 20 hours in LiCl. In both, the characteristic entodermal exogastrular pattern and a slight polar ectodermal pattern appear. Figure 3, *E* is an example of the great entodermal elongation often appearing in exogastrulae of *Patiria*.

Exentogastrulae have been observed very generally in studies of echinoderm exogastrulation. They seem to occur more frequntly in *Patiria* than in the echinoids, but this may be merely a matter of degrees of differential inhibition and recovery or development of differential tolerance to the inhibiting agent. Figures 3, *F–I* are

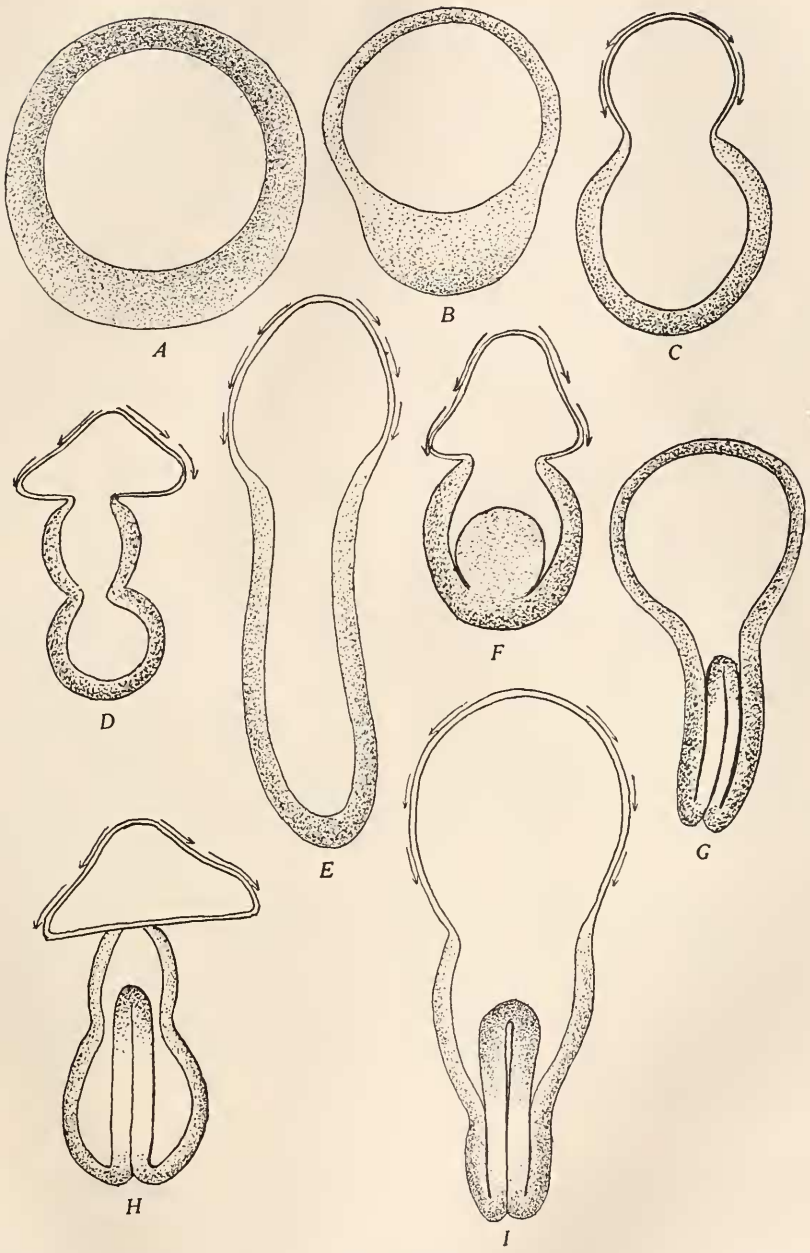

FIGURE 3. Oxidation patterns of *Patiria miniata: A,* normal blastula; *B-E,* different degrees of exogastrulation; *F-I,* exentogastrulae; all LiCl forms, further data in text.

examples of *Patiria* exentogastrulae in material 2–3 days in water after 20 hours in LiCl *M*/50. This concentration of LiCl and period of exposure did not prevent various degrees of differential recovery and an invagination of a part of the entoderm after return to water. In Figure 3, *F* the oxidation gradient pattern of the evaginated part of the entoderm is like that of other exogastrulae, a polar gradient decreasing from the tip of the evaginated region and a cell-wall gradient decreasing from the external surface inward. The invaginated part of the entoderm is a solid mass with a very slight gradient pattern. The most interesting feature of this pattern is that the slight differential present decreases from the tip of the invaginated mass and from its blastocoelar surface inward, at least in the part farthest invaginated. Figures 3, *G*, *H* and *I* are exentogastrulae with polar oxidation gradient decreasing from the tip, and the cell-wall gradient from the external surface of the evaginated part inward. In each of these exogastrulae the elongated invaginated part of the entoderm shows an oxidation gradient decreasing from its tip and in the cell-wall from the blastocoelar surface inward, like the cell-wall gradient of normal development. This gradient pattern did not extend over the entire length of the invaginated entoderm. In *G* and *I* this may have been due to the crowding of evaginated and invaginated parts of the entoderm and failure of indophenol agents to reach the more basal invaginated regions. However, in *H* there is no crowding of the two parts. It appears probable that this re-reversal of the cell-wall gradient pattern requires a certain length of time and that it takes place progressively from the tip along the invaginated part of the entoderm. The invagination and elongation indicate presence of a polar gradient in the invaginating region with its "high" end at the tip. It is also possible that the re-reversal may occur only at the higher polar gradient levels of the invaginated region; lower levels may not recover sufficiently to react in this way. Many years ago, and in numerous cases more recently, differential recovery after inhibition has been observed at higher gradient levels and persisting differential inhibition at lower gradient levels of the same echinoderm larva. In these exentogastrulae of Figures 3, *F–I* the ectoderm is thick enough only in *G* to show a cell-wall gradient decreasing from the blastocoel to the exterior, as well as a polar gradient, decreasing from the apical region. In the other figures only a slight polar gradient is distinguishable in the thin ectoderm.

Patiria, perhaps even more than the other available echinoderms, will undoubtedly repay further study of exogastrulation with different inhibiting agents. The data of the present paper on *Patiria* are by no means final, but, since they agree with the data on echinoids as regards the relation of the cell-wall oxidation gradient to exogastrulation, they are presented merely as part of the evidence that may perhaps throw some light on the physiology of exogastrulation.

Discussion and Conclusions

Earlier studies of redox indicator patterns in echinoderm eggs and early developmental stages have demonstrated the presence of a polar oxidation gradient decreasing basipetally from the apical region in normal development. Also a new oxidation gradient appears in the invaginating entoderm, with decrease from the entodermal tip (Child, 1941a, 1941b; 1944, 1953b). This paper gives further evidence for the existence of these patterns and demonstrates their presence in exogastrulae, except when the ectodermal pattern is completely obliterated by the inhibiting agent.

In the earliest use of indicators on echinoderm developmental stages, only intracellular reduction of vital dyes with external oxygen decrease was considered. With this procedure a reduction gradient through the cell-wall of blastulae and early gastrulae was observed (Child, 1936a, 1936b). It was assumed, without further evidence, that this cell-wall gradient pattern, decreasing from the blastocoel outward, was merely an incidental result of lower oxygen content in the blastocoel than outside, in consequence of oxygen uptake by the cells of the cell-wall or the parts of these cells adjoining the blastocoel, at a higher rate than diffusion of oxygen inward from outside.

Presence of a cell-wall oxidation gradient was of course not recognized in these studies of differential reduction. However, it is now evident that an oxidation gradient, as well as a reduction gradient, is present in the cell-wall of the normal blastula and early gastrula and probably later, and that both gradients decrease from the blastocoel outward. In view of the presence of this oxidation gradient decreasing from the blastocoel outward, there appears to be no adequate basis for the earlier suggestion that the cell-wall reduction gradient, also decreasing from the blastocoel outward, results from lower oxygen content in the blastocoel than outside. That suggestion was never more than a hypothesis to account for the cell-wall reduction gradient. Actually nothing is known concerning oxygen content in the blastocoel, as compared with outside, but it appears improbable that the cell-wall oxidation gradient decreases from a region of lower, to one of higher, oxygen content. The cell-wall oxidation gradient is a feature of normal development; the reduction gradient appears only after oxygen decrease, either by oxygen uptake of the individual sealed in a small volume of liquid, or by use of a reducing agent. Perhaps under these conditions oxygen content in the blastocoel may become lower than outside in consequence of greater oxidase activity adjoining the blastocoel. The two cell-wall indicator gradients, both decreasing from the blastocoel outward, can be demonstrated in the same individual, though of course not at the same time. The oxidation gradient appears under natural conditions, the reduction gradient only after oxygen decrease.

In the evaginated entoderm of the exogastrula the cell-wall oxidation gradient decreases from the external surface inward, *i.e.*, it is reversed, as compared with the normal individual. This reversal has been observed only in that part of the exogastrula which evaginates as entoderm. This may vary widely in extent in either direction from the normal region of prospective entoderm. It may include only the most basal part of this region, as in Figure 2, *H*. In the earliest study of gradient pattern in exogastrulae it was also shown that in *S. purpuratus* and *S. franciscanus*, with delay of exposure to LiCl to late blastula stages, only the most basal part of the prospective entodermal region evaginated and developed as entoderm, or evagination might not occur, and entoderm might not be clearly distinguishable from ectoderm. At this stage of echinoid development entodermal activity is increasing and it is more inhibited than ectoderm. Incidentally, it is a question of some interest whether at this stage inhibited prospective entoderm is ectodermized. Certainly all degrees of apparent ectodermization appear under these conditions (Child, 1936b, Figs. 36–44). Similar restriction of evagination occurs in *Dendraster* under similar conditions (Child, 1940, Figs. 74–76). From forms of this character, often with even smaller evaginated entoderms with reversals of

oxidation gradients, at one extreme of exogastrulation, the region of evaginated entoderm and gradient reversal may extend in all degrees, not only over all of the prospective entodermal region, but also into the region of prospective ectoderm, until only a small knob of ectoderm remains in the apical region (Fig. 1, *I* above) or until the entire body is entodermized.[4] It is evident from the numerous studies on exogastrulation that, with exposure to the exogastrulating agent beginning in early developmental stages, the part of the individual undergoing evagination as entoderm in general extends farther apically as degree of effect of the agent increases. In a given lot of eggs, particularly if they are from different females, a wide range of sensitivity to exogastrulating action may occur. Consequently a wide range in the part of the animal undergoing evagination and reversal of the cell-wall oxidation gradient may occur in a single concentration of the agent. It is evident from these data that there is no regional difference in prospective ectoderm and prospective entoderm in early development which a single inhibiting agent cannot completely obliterate. It is also evident that in early stages lability increases basipetally, though perhaps not uniformly.

In normal pregastrular stages ectodermal development is more rapid and its gradient pattern soon becomes less labile than that of prospective entoderm, the apical region, the "high" end of the polar gradient, the least labile of all. It probably also becomes morphologically different from entoderm, as its further development suggests. With the gradual decrease in thickness of the ectoderm it becomes increasingly difficult to determine whether the cell-wall gradient persists in it. The basipetal differential in ectodermal lability apparently increases as the polar gradient differential increases from early stages onward.

It has seemed to be very generally true that the degree of inhibition necessary for entodermization of prospective ectoderm increases acropetally. It is necessary, however, to call attention here to certain forms of *S. purpuratus* appearing with inhibition by sodium azide, certain constituents of tobacco smoke and even with extreme crowding in water and in some cases with LiCl. In certain lots of material of this species all degrees of alteration of the apical ectodermal region from mere thickenings to outgrowths which are identical in appearance with evaginated entoderm at the basal pole of exogastrulae or in forms with invaginated basal entoderm appeared in large numbers in many containers. These apical outgrowths in many cases developed three segments, exactly like the basal entoderm (Child, 1948). In the first study of indicator patterns in exogastrulae somewhat smaller apical outgrowths, though often with three segments like basal entoderm, appeared very frequently with LiCl inhibition but were not included in published data, as it was desired to obtain further evidence as regards their occurrence. At present the only suggestion that seems to account for these forms is that the action of the agents was effective so early in development that alteration of the apical region occurred before its lability decreased appreciably. As the "high" end of the polar gradient it was more susceptible to inhibition and alteration than other levels of this gradient. If the agents were sufficiently effective very early in development, it seems possible that alteration and even entodermization of the apical region might occur without much effect on other levels of prospective ectoderm. *S. purpuratus* is in general more

[4] For earlier figures of this range of forms of exogastrulae see MacArthur, 1924, particularly Figure 2; Child, 1936b, Figures 1–5, 1940, various figures.

susceptible to inhibiting agents than *Dendraster* or *Patiria*. In *Dendraster* slight degrees of thickening and alteration of the apical region have been occasionally observed, but nothing that could be regarded as actual entodermization. Also only slight apical alterations have been observed in more recent *S. purpuratus* material. It is possible that the material of the 1948 paper was for some reason unusually susceptible or that the polar differential of early stages was greater than usual. Long experience with echinoderm material suggests that different lots may differ considerably in degree of reaction to external agents and in differential effects.

According to Figures 1, *J, K, L,* 2, *B, C, D,* and 3, *B* and *G,* above, there is no reversal in the cell-wall oxidation gradient as long as its ectodermal character persists. Apparently reversal occurs only when it is entodermized and takes part in evagination. The occurrence of reversal in the cell-wall oxidation gradient in evaginated entoderm raises what are perhaps the most interesting questions associated with exogastrulation. What determines reversal of this gradient? Does this reversal constitute a reversal of the physiological polarity of the entoderm cells or of the entodermal region in which it occurs? And finally, does the reversal determine reversal in the direction of entodermal growth, *i.e.*, evagination instead of invagination, or is it merely an incident or a result of an evagination determined in some unknown manner?

Considering first the question, how reversal occurs, it is evident that the cell-wall gradient represents a relatively slight differential. In a cell-wall only one cell thick it occurs between inner and outer ends of single cells. In solid entodermal masses it may involve multicellular regions. It is most clearly visible in the earlier stages of intracellular oxidation. As intracellular concentration of indophenol or oxidized dyes increases, it becomes difficult or impossible to distinguish it. In the presence of differentially inhibiting, exogastrulating agents this differential is undoubtedly decreased or perhaps completely obliterated with less inhibition than the polar pattern with much greater differential from apical to basal regions. The blastocoelar end of the cell-wall gradient, the region of higher oxidase activity, will undergo the greatest decrease. In various series it was observed in all three species that under inhibiting conditions the cell-wall gradient was very slight or could not be distinguished in many individuals which remained in blastula stages without definite invagination or evagination of entoderm.[5] As regards the intracellular conditions determining reversal of the cell-wall gradient in evaginating entoderm only suggestion is at present possible. Following decrease or obliteration of the original cell-wall gradient of evaginating entoderm, it appears highly probable that diffusion of oxygen inward, together with oxygen uptake of the cells, will establish a new gradient decreasing from the external surface. Less oxygen will reach those parts of the cells farthest from the external surface than those nearer the exterior.

[5] More attention was given to this point in *S. purpuratus* material than in the other forms. In laboratory records concerning this species decrease or absence of the entodermal cell-wall gradient was noted in blastulae 20 hours in LiCl *M*/20, two days in *M*/50, two days in *M*/60, in a lot with extreme crowding in water and in a lot one day in water after one day in sodium azide *M*/500. In *Dendraster* almost complete absence of this gradient was noted after 20 hours in azide *M*/600, after 20 hours in LiCl *M*/50, and in blastulae still living after a day in water following 20 hours in azide *M*/250. In various other lots presence of this gradient was uncertain but this was not specially recorded. These are believed to be cases in which the original cell-wall gradient was in process of being obliterated by the exogastrulating agent.

In various other organisms, particularly among the hydroids, an oxygen differential from the free surface, perhaps also an opposed carbon dioxide differential from the surface in contact with the substrate are apparently the factors determining a new polar gradient pattern and various other modifications of morphological pattern (Child, 1941a, pp. 413–420; also pp. 425–6 and Fig. 144). Moreover, oxygen may be an important factor in the experimental determination of ventrodorsality in *Dendraster* (Pease, 1941, 1942a, 1942b). Echinoderm material exposed to exogastrulating agents is usually returned to water before actual evagination occurs. Except in cases of extreme inhibition, this permits more or less differential recovery and still greater opportunity for determination of a new cell-wall gradient. It appears possible, however, that even without return to water, diffusion of oxygen into the cell-wall, together with the oxygen uptake of the cells, though oxygen uptake is less than under natural conditions, may determine a new gradient, decreasing from the external surface inward.

As regards the question of reversal of entodermal polarity by the reversal of the cell-wall gradient, it is to be noted first that physiological axiate patterns of morphogenesis and gradient pattern have been shown to be closely associated in many organisms, both in embryonic development and in reconstitution in later life. When ventrodorsal gradient pattern is obliterated by inhibiting agents in early echinoderm development, completely radial forms develop, like most of the exogastrulae in the figures of this paper. When gradient patterns are almost or entirely obliterated by an external agent morphogenesis is almost or completely absent (*e.g.*, Child, 1948, Figs. 79–85).[6] In the case of echinoderm exogastrulation there seems at present to be no reason for doubting that reversal of the cell-wall gradient reverses, partially or completely, entodermal polarity.

In view of the very general association of gradient pattern and course and character of development it appears highly probable that this reversal of the cell-wall gradient and entodermal evagination instead of invagination are directly associated. The entoderm invaginates when it possesses a certain cell-wall gradient. When this pattern is reversed it is highly probable that it must evaginate.

In the case of exogastrulation resulting from exposure to a very low temperature in earlier stages with transfer later to a room temperature of about 20° C. (Fig. 1, *C* above), the low temperature during the earlier stages evidently acts like other exogastrulating agents and decreases or obliterates the cell-wall gradient. On transfer to the much higher temperature, the relation between the greatly increased oxygen uptake and the diffusion of oxygen inward probably determines the reversal. The exogastrulae produced in this way in *Dendraster* are almost entirely of the less extreme types, with ectoderm approaching or attaining pluteus differentiation, *i.e.*, ectodermal recovery is almost complete, but reversal of the entodermal cell-wall gradient and evagination occur.

One other point remains to be considered. With certain degrees of differential inhibition exentogastrulae appear more or less frequently, usually with the less

[6] For another case of obliteration of gradient pattern and of morphogenesis see Child, 1941a, pp. 167–69, Figure 57 and pp. 425–6 and Figure 144. On the other hand, new patterns with a variety of symmetries in addition to the polar pattern develop in the reconstitution of isolated pieces of *Corymorpha* in relation to the differential originating between the surface in contact with the glass of the container and the parts freely exposed to the water (Child, 1941a; pp. 413–420, Figs. 141 and 142).

extreme degrees of inhibition and after return to water and differential recovery. They seem to occur more frequently in *Patiria* than in the echinoids and in many of these forms the entire length of the entoderm becomes very great, as in Figures 3, *G*, *H*, and *I*. Moreover, in these cases there seems to be a re-reversal of the cell-wall gradient in the terminal region of the invaginated part. Except for this terminal region the invaginated part may be crowded inside of the evaginated part and absence of any gradient pattern in it may be due merely to failure of the redox agents to reach it (Figs. 3, *G* and *I*), though in Figure 3, *H* the cell-wall gradient is evident only in the terminal region, and in that case there is no crowding. All of the exentogastrulae of these figures and various others were returned to water after 20 hours in LiCl *M*/50 at room temperature.

The change from evagination to invagination of the entoderm is the feature of greatest interest in these forms. As regards conditions determining this change, it is suggested that the reversal of the cell-wall gradient may not be quite complete in the evaginated entodermal tip, that some enzymatic or other trace of the original gradient may persist but may be obscured or overlaid by the gradient decreasing from the external surface. With differential recovery after return to water the tip of the evaginated entoderm recovers most rapidly and most completely, and the original cell-wall gradient may be to some degree reactivated and become sufficiently effective to bring about the beginning of invagination in the entodermal tip. If the beginning of invagination is determined in this way increase in the differential of this re-reversal of the cell-wall gradient may be expected to occur, at least in the invaginating tip. In the blastocoel the relation between diffusion of oxygen from the blastocoel into the cell-wall and oxygen uptake of the cells may be regarded as the factor concerned in this increase. In the terminal regions of the invaginated entoderms of Figures 3, *G*, *H*, and *I* and even in the invaginated entodermal mass of Figure 3, *F* this pattern is distinguishable.

With increase in concentration of the exogastrulating agent or increase in length of exposure period exentogastrulae decrease in frequency and with sufficient inhibition do not appear at all, even after return to water. This relation to concentration of agent and exposure period seems to indicate complete obliteration or destruction of any basis or substrate for the original cell-wall gradient. The reversed gradient pattern has apparently become irreversible in these cases.

Figures 3, *F–I* show only later or final stages of exentogastrulae. It has not been possible thus far to identify the beginnings of exentogastrulation, *i.e.*, the pattern of the entoderm at the critical point of the change from evagination to invagination. The indicators injure or finally kill and it is not certain that particular individuals which may suggest a change in gradient pattern would go on to definite exentogastrulation if they remained alive.

In the entexogastrula with invagination followed by evagination, the inhibiting conditions are at first not sufficient to determine evagination of entoderm but become effective later. Their occurrence may be determined by delay of exposure to the agent to slightly later developmental stages than those ordinarily used for exogastrulation or by increasing concentration of the agent after an initial exposure. In these there is merely the single change from invagination to evagination of entoderm.

Further studies of *Patiria* exogastrulation in 1953 indicate that at least some of those forms with both evaginated and invaginated entoderms are entexogastrulae,

rather than exentogastrulae. In those cases some degree of entodermal invagination is followed by evagination as effect of the inhibiting agent increases. Both invaginated and evaginated entoderm may continue to increase in length. In entexogastrulae the cell-wall gradient of the tip of the invaginated entoderm is not a "re-reversal" of pattern, but a persistence or a recovery of the original pattern, with decrease from the blastocoelar surface.

Summary

1. A modification of the Nadi or indophenol reaction, using very low concentrations of agents, and intracellular oxidation of dyes reduced by sodium hydrosulphite, demonstrates the existence of a cell-wall oxidation gradient, decreasing from the blastocoel outward and from the apical region basipetally, in normal blastulae and early gastrulae of *Strongylocentrotus purpuratus, Dendraster excentricus* and *Patiria miniata*.

2. In the evaginated entoderm of exogastrulae, derived either from the original prospective entoderm or from entodermized ectoderm, the cell-wall gradient is reversed in direction, decreasing from the external surface toward the blastocoel.

3. The cell-wall oxidation gradient of ectoderm which persists as ectoderm does not undergo reversal, but as the cell-wall decreases in thickness, it becomes difficult or impossible to determine whether it persists.

4. Reversal of the cell-wall oxidation gradient by an exogastrulating agent is regarded as resulting from two factors: first, more or less complete obliteration of the original gradient by differential inhibition; second, from establishment of the reversed gradient by the relation between diffusion of oxygen inward from the external entodermal surface and the oxygen uptake of the entodermal cells.

5. It is suggested that this reversal of the oxidation gradient pattern reverses the polarity of the entoderm, and the reversed polarity determines evagination instead of invagination.

6. In exentogastrulae entodermal evagination is followed by invagination. In some of the exentogastrulae appearing in *Patiria* material a "re-reversal" of the cell-wall gradient occurs in the terminal region of the invaginated part of the entoderm. It is suggested that in the exentogastrulae some trace of the original cell-wall gradient persists, although the visible gradient is reversed. After differential recovery, following return to water, some degree of reactivation of the original gradient in the cell-wall of the entodermal tip, the region of most rapid and most complete recovery, determines the beginning of invagination, and conditions in the blastocoel bring about further reactivation in some individuals. Exentogastrulae decrease in frequency or do not appear at all with increasing degrees of inhibition, presumably because the original cell-wall gradient has been completely obliterated. In entexogastrulae there is no "re-reversal" of the cell-wall gradient but merely persistence or recovery of the original gradient.

LITERATURE CITED

Child, C. M., 1936a. Differential reduction of vital dyes in the early development of echinoderms. *Arch. f. Entw.*, **135**: 426–456.

Child, C. M., 1936b. A contribution to the physiology of exogastrulation in echinoderms. *Arch. f. Entw.*, **135**: 457–493.

CHILD, C. M., 1940. Lithium and echinoderm exogastrulation. *Physiol. Zool.*, **13**: 4–42.

CHILD, C. M., 1941a. Patterns and problems of development. University of Chicago Press, Chicago, Illinois.

CHILD, C. M., 1941b. Formation and reduction of indophenol blue in development of an echinoderm. *Proc. Nat. Acad. Sci.*, **27**: 523–528.

CHILD, C. M., 1944. Developmental pattern in the starfish *Patiria miniata*, as indicated by indophenol. *Physiol. Zool.*, **17**: 129–151.

CHILD, C. M., 1948. Exogastrulation by sodium azide and other inhibiting conditions in *Strongylocentrotus purpuratus*. *J. Exp. Zool.*, **107**: 1–38.

CHILD, C. M., 1953a. Exogastrulation and differential cell dissociation by sodium azide in *Dendraster excentricus* and *Patiria miniata*. *Physiol Zool.*, **26**: 28–58.

CHILD, C. M., 1953b. Indicator gradient pattern in oöcytes and early developmental stages of echinoderms: a reexamination. *Biol. Bull.*, **104**: 12–27.

MACARTHUR, J. W., 1924. An experimental study and a physiological interpretation of exogastrulation and related modifications in echinoderm embryos. *Biol. Bull.*, **46**: 60–87.

MOORE, A. R., 1952. The process of gastrulation in trypsin embryos of *Dendraster excentricus*. *J. Exp. Zool.*, **119**: 37–46.

PEASE, D. C., 1941. Echinoderm bilateral determination in chemical concentration gradients. I. The effects of cyanide, ferricyanide, iodoacetate, picrate, dinitrophenol, urethane, iodine, malonate etc. *J. Exp. Zool.*, **86**: 381–404.

PEASE, D. C., 1942a. Echinoderm bilateral determination in chemical concentration gradients. II. The effects of azide, pilocarpine, pyocyanine, diamine, cysteine, glutathione and lithium. *J. Exp. Zool.*, **89**: 329–345.

PEASE, D. C., 1942b. Echinoderm bilateral determination in chemical concentration gradients. III. The effects of carbon monoxide and other gases. *J. Exp. Zool.*, **89**: 347–386.